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Correlation between Genotypic Diversity, Lipooligosaccharide Gene Locus Class Variation, and Caco-2 Cell Invasion Potential of *Campylobacter jejuni* Isolates from Chicken Meat and Humans: Contribution to Virulotyping▽

Ihab Habib,[1,6]* Rogier Louwen,[2] Mieke Uyttendaele,[3] Kurt Houf,[1] Olivier Vandenberg,[4] Edward E. Nieuwenhuis,[2] William G. Miller,[5] Alex van Belkum,[2] and Lieven De Zutter[1]

Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke,[1] Laboratory of Food Microbiology and Food Preservation, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent,[3] and National Reference Centre for Enteric Campylobacters, Department of Microbiology, Saint-Pierre University Hospital, Rue Haute 322, B-1000 Brussels,[4] Belgium; Department of Medical Microbiology and Infectious Diseases, Erasmus MC, University Medical Centre,'s Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands[2]; Produce Safety and Microbiology Research Unit, U.S. Department of Agriculture (USDA), ARS, WRRC, 800 Buchanan Street, Albany, California 94710[5]; and Food Hygiene and Control Division, High Institute of Public Health (HIPH), Alexandria University, 165 El-Horrya Avenue, Alexandria, Egypt[6]

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Significant interest in studying the lipooligosaccharide (LOS) of *Campylobacter jejuni* has stemmed from its potential role in postinfection paralytic disorders. In this study we present the results of PCR screening of five LOS locus classes (A, B, C, D, and E) for a collection of 116 *C. jejuni* isolates from chicken meat ($n = 76$) and sporadic human cases of diarrhea ($n = 40$). We correlated LOS classes with clonal complexes (CC) assigned by multilocus sequence typing (MLST). Finally, we evaluated the invasion potential of a panel of 52 of these *C. jejuni* isolates for Caco-2 cells. PCR screening showed that 87.1% (101/116) of isolates could be assigned to LOS class A, B, C, D, or E. Concordance between LOS classes and certain MLST CC was revealed. The majority (85.7% [24/28]) of *C. jejuni* isolates grouped in CC-21 were shown to express LOS locus class C. The invasion potential of *C. jejuni* isolates possessing sialylated LOS ($n = 29$; classes A, B, and C) for Caco-2 cells was significantly higher ($P < 0.0001$) than that of *C. jejuni* isolates with nonsialylated LOS ($n = 23$; classes D and E). There was no significant difference in invasiveness between chicken meat and human isolates. However, *C. jejuni* isolates assigned to CC-206 (correlated with LOS class B) or CC-21 (correlated with LOS class C) showed statistically significantly higher levels of invasion than isolates from other CC. Correlation between LOS classes and CC was further confirmed by pulsed-field gel electrophoresis. The present study reveals a correlation between genotypic diversity and LOS locus classes of *C. jejuni*. We showed that simple PCR screening for *C. jejuni* LOS classes could reliably predict certain MLST CC and add to the interpretation of molecular-typing results. Our study corroborates that sialylation of LOS is advantageous for *C. jejuni* fitness and virulence in different hosts. The modulation of cell surface carbohydrate structure could enhance the ability of *C. jejuni* to adapt to or survive in a host.

Campylobacter jejuni is an important human enteric pathogen worldwide (3, 7, 26). Infected humans exhibit a range of clinical spectra, from mild, watery diarrhea to severe inflammatory diarrhea (28). Factors influencing the virulence of *C. jejuni* include motility, chemotaxis, the ability to adhere to and invade intestinal cells, intracellular survival, and toxin production (28, 30, 52). Besides its role in human enteric illnesses, *C. jejuni* is a predominant infectious trigger of acute postinfectious neuropathies, such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) (1). Significant interest in studying the structure and biosynthesis of the core lipooligosaccharide (LOS) of *C. jejuni* has resulted from its potential role in these paralytic disorders. Many studies have now provided convincing evidence that molecular mimicry between *C. jejuni* LOS and gangliosides in human peripheral nerve tissue plays an important causal role in the pathogenesis of GBS/MFS (16, 17, 19, 21).

Initial comparative studies of *C. jejuni* LOS structure and the corresponding DNA sequences of the LOS biosynthesis loci identified eight different LOS locus classes. Three of these classes, A, B, and C, harbor sialyltransferase genes involved in incorporating sialic acid into the LOS (42). Sialylation of the LOS core was found to be associated with ganglioside mimicry and also to affect immunogenicity and serum resistance (21). Recently, Parker et al. (43) identified 11 additional LOS classes on the basis of the sequence at the LOS biosynthesis locus. Their investigation also suggested that the LOS loci of *C. jejuni* strains are hot spots for genetic exchange, which can lead to mosaicism.

Despite evidence on locus variation within *C. jejuni* LOS classes, PCR-based screening of a collection of 123 clinical and

* Corresponding author. Mailing address: Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: 32 09 264 73 41. Fax: 32 09 264 74 91. E-mail: ihab.habib@ugent.be.

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environmental strains showed that almost 60% of *C. jejuni* strains belong to class A, B, or C (42). Additionally, Godschalk et al. (16) found that 53% (9/17) of GBS-associated *C. jejuni* strains possessed LOS of class A, while 64% (35/55) of the non-GBS-associated isolates possessed LOS of class A, B, or C, and 62% (13/21) of enteritis-associated *Campylobacter* strains expressed LOS of class A, B, or C, as well. This relative representation of sialylated LOS classes A, B, and C was hypothesized to be advantageous for *C. jejuni* in the colonization and infection of various hosts (42, 49). Recently, Louwen et al. (34) demonstrated that *C. jejuni* strains possessing sialylated LOS (class A, B, or C) invade Caco-2 cells significantly better than nonsialylated strains (with class D or E). Knockout mutagenesis of the LOS sialyltransferase Cst-II in three *C. jejuni* strains revealed a significant reduction in the invasion potentials of the mutant strains (34). The possible role of LOS in adhesion and invasion was previously highlighted in the work of Perera et al. (44) and Kanipes et al. (29), where a *C. jejuni waaF* mutant strain showed significant reductions in levels of adherence to and invasion of INT-407 cells.

LOS class diversity in *C. jejuni* strains isolated from chicken meat, an important source of human campylobacteriosis (6, 7, 26), has hardly been studied at all. In addition, the role of LOS class variation in the invasion potential of *C. jejuni* strains from chicken meat still needs to be explored. The epidemiological relevance of *C. jejuni* LOS gene screening can be further elaborated by correlating its results with results from other molecular-typing tools (e.g., multilocus sequence typing [MLST] and pulsed-field gel electrophoresis [PFGE]). In the present study, we screened a diverse collection of *C. jejuni* isolates, from consumer-packaged chicken meats and from sporadic human cases of diarrhea, by PCR for five LOS classes (A, B, C, D, and E). Then we correlated the LOS classes assigned by PCR screening with the genotypes assigned by PFGE and MLST. Finally, we tested the invasion potentials of a representative subset of *C. jejuni* isolates in relation to their LOS classes and genotypic diversity.

MATERIALS AND METHODS

Isolate collection and growth conditions. All chicken meat ($n = 76$) and human ($n = 40$) isolates were identified as *C. jejuni* by using multiplex PCR as described by Vandamme et al. (50). The food-related bacterial collection consisted of 76 *C. jejuni* isolates from 74 chicken meat preparation samples. The term "chicken meat preparation" refers to portioned, cut, or minced meat to which other ingredients (e.g., salt, spices, seasoning mix, marinade, or sauce) may have been added, though the cut surface retains the characteristics of raw meat (2). The chicken meat samples were from five Belgian companies and were collected in a survey between February and November 2007 (22). In addition, 40 clinical *C. jejuni* isolates were also investigated. Strains in the human collection were isolated from the stool specimens of 39 patients admitted with sporadic cases of diarrhea and were provided by the same Belgian hospital laboratory in Brussels. Human isolates were cultured over the period from May to September 2007, and related clinical data were supplied. Table 1 provides details on the origins and sampling dates for all isolates, in addition to data on the sources and processing batches of chicken meat samples.

The isolate collection was stored at −80°C in sterile full horse blood (E & O Laboratories, Bonnybridge, United Kingdom) and had been minimally subcultured before storage and subsequent testing. When required, isolates were cultured from the frozen stock for 24 h on blood agar plates (Muller-Hinton agar base CM337 [Oxoid, Basingstoke, United Kingdom] supplemented with 5% [vol/vol] full horse blood [E & O Laboratories]) under a microaerobic atmosphere at 37°C.

PCR screening of LOS locus classes. DNA was prepared by alkaline lysis as described previously (10). Primer sets specific for LOS locus classes A/B, B, C, D, and E were used (16). LOS class A isolates were distinguished from class B isolates as those amplified by PCR primer A/B but not after successive screening by a class B-specific primer. PCR assays were performed using an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA) with a touchdown program consisting of an initial denaturation step of 5 min at 94°C; 10 cycles of 1 min at 94°C, 1 min at 60°C (with the temperature initially reduced by 1°C per cycle until it reached 50°C), and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. PCRs were performed in a 25-μl volume; each reaction mixture contained 2 μl of DNA template and a PCR mixture consisting of 1× buffer, 200 μM each deoxynucleoside triphosphate, 50 pmol of each PCR primer, 3 mM $MgCl_2$, and 2 U of *Taq* polymerase per reaction (final concentrations). All PCR reagents were from Invitrogen, Merelbeke, Belgium. DNAs from GBS/MFS- and enteritis-associated *C. jejuni* strains for which the LOS loci had been identified previously (34) were used as PCR-positive controls.

MLST. All *C. jejuni* isolates were characterized by MLST on the basis of primers for seven gene targets for each isolate (*aspA* [encoding aspartase A], *glnA* [glutamine synthase], *gltA* [citrate synthase], *glyA* [serine hydroxymethyltransferase], *pgm* [phosphoglucomutase], *tkt* [transketolase], and *uncA* [ATP synthase alpha subunit]) under conditions described previously (11, 35). All allelic sequences were queried against the *C. jejuni* MLST database (http://pubmlst.org/campylobacter/), developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford. Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the *C. jejuni* MLST database and assigned new numbers.

PFGE. PFGE was performed using SmaI-digested fragments of bacterial chromosomal DNA as previously described (45). Gel patterns were analyzed using GelCompar software (Applied Maths, Kortrijk, Belgium) with the band tolerance set at 1.5% (41).

Invasion assay. A panel of 52 *C. jejuni* isolates from chicken meat ($n = 30$) and from human enteritis cases ($n = 22$) was selected for the invasion assay. The 52 isolates were randomly selected in relation to their PCR-assigned LOS locus classes, as follows: 12 isolates each of classes B, C, and E, 11 isolates of class D, and all 5 isolates identified as class A. The gentamicin protection assay used in this study was the same as that described by Louwen et al. (2008) (34). Briefly, Caco-2 cells were seeded and grown to confluence (~5.0×10^6 cells) in 6-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Monolayers were incubated with *C. jejuni* at a multiplicity of infection of 100:1 for 4 h at 37°C under a 5% CO_2 atmosphere. Cells were then washed with prewarmed Dulbecco's modified Eagle's medium (Invitrogen, Breda, The Netherlands) and incubated for another 2 h in 2 ml Dulbecco's modified Eagle's medium containing gentamicin (480 μg ml^{-1}) to kill extracellular bacteria. After the gentamicin kill period, the infected monolayers were washed three times with Hanks' buffered salt solution (Invitrogen, Breda, The Netherlands) and lysed with 0.1% Triton X-100 (Cornell, Philadelphia, PA) in phosphate-buffered saline (Invitrogen, Breda, The Netherlands) for 15 min at room temperature to release the intracellular bacteria. The number of viable bacteria released from the cells was assessed after serial 10-fold dilutions of the lysates on blood agar plates (Becton Dickinson, Breda, The Netherlands). Percentages of internalization were calculated, based on four tests per strain, by performing two independent assays, each done in duplicate on separate occasions and by different technicians working in parallel. A Penner serotype *C. jejuni* reference strain (P4; low invasiveness [34]) was used as an internal control strain to account for interexperimental variation.

PCR screening of virulence-related genes. The 52 *C. jejuni* isolates selected for the invasion assay were also screened for the presence of certain adhesion-, invasion-, and toxin-related genes. Previously published PCR primers and conditions were used for the detection of *ceuE* (20), *cadF* (32), *ciaB* (9), *pldA* (9), *cdtA* (24), *cdtB* (9), and *cdtC* (9).

Statistical analysis. Correlation between categorical independent variables (isolates' origins, LOS classes, and MLST clonal complexes) and the invasion phenotype (the dependent variable) was tested. The dependent variable in this analysis is estimated as the number of *C. jejuni* CFU on blood agar plates after plating from Caco-2 monolayer lysates. The CFU count on agar plates follows a Poisson distribution, the distribution used to fit counts (≥ 0) of events that should be randomly distributed in space and time. Thus, the analysis was conducted using generalized linear models, starting with Poisson regression analysis. In case of extra-Poisson variation, negative binomial regression was used to account for the overdispersion in the data. Differences in the number of *C. jejuni* isolates between comparison groups (e.g., differences in numbers of isolates per clonal complex) were accounted for by applying frequency-weighting procedures. Model analysis was performed and fitted in Stata statistical software, version 8.0. (48).

TABLE 1. Identification of *C. jejuni* isolates included in the study[a]

LOS class	Origin	Isolate[b]	Isolation date	Sequence type[c]	Clonal complex[d]	Meat sample no.[e]
A (n = 5)	Chicken (n = 4)	272-2	3 May 2007	257	257	M-23
		187-1	11 Apr. 2007	3550*	21	F-4
		157-1	3 Apr. 2007	704	NA	P-8
		501-3	21 Aug. 2007	883	21	F-11
	Human (n = 1)	R357	8 Sept. 2007	262	21	
B (n = 26)	Chicken (n = 14)	442-1	29 June 2007	122	206	M-32
		272-1	3 May 2007	257	257	M-22
		353-2	6 June 2007	305	574	M-24
		490-1	14 Aug. 2007	305	574	M-V
		419-1	22 June 2007	3548*	NA	M-28
		422-2	22 June 2007	3548*	NA	M-30
		344-1	1 June 2007	42	42	P-13
		353-1	6 June 2007	464	NA	M-24
		538	10 Sept. 2007	48	48	P-17
		307-1	22 May 2007	572	206	C-8
		285-1	8 May 2007	572	206	N-6
		134-1	27 Mar. 2007	572	206	C-4
		40-2	27 Feb. 2007	572	206	C-1
		495-3	14 Aug. 2007	572	206	C-12
	Human (n = 12)	D801	10 July 2007	1377	42	
		E541	10 Aug. 2007	42	42	
		E540	6 Aug. 2007	1724	460	
		G373	31 July 2007	572	206	
		H397	8 Aug. 2007	572	206	
		J152	28 Aug. 2007	122	206	
		K328	19 Sept. 2007	3552*	446	
		L406	9 July 2007	1377	42	
		L407	9 July 2007	3553*	NA	
		O197	26 Sept. 2007	607	607	
		P243	27 July 2007	3554*	NA	
		Z145	1 May 2007	450	446	
C (n = 30)	Chicken (n = 22)	204-1	12 Apr. 2007	19	21	M-18
		530-1	3 Sept. 2007	19	21	F-12
		201-3	12 Apr. 2007	2037	NA	M-16
		39-2	27 Feb. 2007	2324	NA	F-2
		271-1	3 May 2007	25	45	M-22
		240	24 Apr. 2007	3128	21	C-6
		654	20 Nov. 2007	3544*	NA	M-45
		214-3	17 Apr. 2007	3546*	NA	N-5
		429-2	26 June 2007	44	21	C-11
		109-1	20 Mar. 2007	50	21	N-2
		559	18 Sept. 2007	50	21	M-36
		629	6 Nov. 2007	50	21	C-V
		79E1	13 Mar. 2007	50	21	F-3
		135-1	27 Mar. 2007	50	21	C-5
		202-1	12 Apr. 2007	50	21	M-17
		221-1	18 Apr. 2007	50	21	P-10
		581	9 Oct. 2007	50	21	F-10
		499-3	21 Aug. 2007	50	21	F-Z
		51-1	6 Mar. 2007	50	21	P-4
		75-1	13 Mar. 2007	50	21	F-3
		408	19 June 2007	53	21	N-7
		357-1	6 June 2007	990	257	M-26
	Human (n = 8)	A842	4 June 2007	1728	21	
		C463	21 Aug. 2007	19	21	
		D807	20 Sept. 2007	53	21	
		G374	30 Sept. 2007	21	21	
		L408	27 July 2007	21	21	
		M766C	8 June 2007	50	21	
		P242	10 July 2007	1728	21	
		V485	6 May 2007	19	21	

Continued on following page

TABLE 1—*Continued*

LOS class	Origin	Isolate[b]	Isolation date	Sequence type[c]	Clonal complex[d]	Meat sample no.[e]
D (n = 21)	Chicken (n = 15)	136-1	27 Mar. 2007	2258	NA	C-5
		2-2	7 Feb. 2007	257	257	F-1
		46-4	1 Mar. 2007	257	257	M-1
		48-1	1 Mar. 2007	257	257	M-3
		158-1	3 Apr. 2007	2883	NA	P-8
		478	10 July 2007	2899	446	P16
		355-1	6 June 2007	354	354	M-25
		356-1	6 June 2007	354	354	M-26
		603-1	23 Oct. 2007	354	354	M-38
		239-1	24 Apr. 2007	354	354	F-6
		537	10 Sept. 2007	45	45	P-17
		498-1	14 Aug. 2007	45	45	C-12
		8-2	15 Feb. 2007	775	52	P-2
		256-1	27 Apr. 2007	990	257	M-19
		199-1	12 Apr. 2007	990	257	M-14
	Human (n = 6)	A841	31 May 2007	354	354	
		C457	11 June 2007	354	354	
		C458	21 June 2007	354	354	
		E539	27 July 2007	969	354	
		T317	13 June 2007	2398	NA	
		V488	12 June 2007	775	52	
E (n = 19)	Chicken (n = 9)	501-1	21 Aug. 2007	2219	45	F-11
		556-2	18 Sept. 2007	2807	443	M-33
		208-1	17 Apr. 2007	443	443	M-19
		268-2	3 May 2007	45	45	M-21
		358	6 June 2007	45	45	M-27
		373E1	8 June 2007	45	45	N-6
		122E	22 Mar. 2007	45	45	P-7
		41-2	27 Feb. 2007	45	45	C-2
		146	28 Mar. 2007	51	443	M-10
	Human (n = 10)	B1093	3 July 2007	583	45	
		B1096	10 Sept. 2007	583	45	
		Z146	22 Aug. 2007	400	353	
		C461	13 July 2007	400	353	
		D803	30 July 2007	137	45	
		D804	30 July 2007	137	45	
		D805	30 July 2007	137	45	
		M768	7 July 2007	443	443	
		R356	24 July 2007	267	283	
		S478	11 Sept. 2007	443	443	
Others (n = 15)	Chicken (n = 12)	393-2	15 June 2007	1759	NA	C-10
		423	22 June 2007	2037	NA	M-31
		6-2	15 Feb. 2007	2037	NA	P-2
		238-2	24 Apr. 2007	2324	NA	F-5
		149	28 Mar. 2007	257	257	M-12
		1-2	7 Feb. 2007	257	257	F-1
		7-2	15 Feb. 2007	257	257	P-2
		560	18 Sept. 2007	3544*	NA	M-37
		652	20 Nov. 2007	3544*	NA	M-43
		121-1	22 Mar. 2007	3544*	NA	P-7
		222-1	18 Apr. 2007	50	21	P-10
		461	3 July 2007	905	NA	N-8
	Human (n = 3)	B1094	2 Aug. 2007	48	48	
		C456	7 Sept. 2007	354	354	
		L404	13 June 2007	3546*	NA	

[a] Isolates from humans (n = 40) and chicken meat (n = 76) are grouped according to their LOS classes, isolation dates, and MLST results.
[b] Isolates 272-2 and 272-1 (classes A and B, respectively) are from the same chicken meat sample. Isolates D803 and D804 (class E) are from the same human sample.
[c] Asterisks indicate novel sequence types, first reported in this collection.
[d] NA, not assigned to a defined clonal complex (MLST online database last accessed in September 2008).
[e] Given as the code for the company of origin-number of the processing batch.

TABLE 2. Comparison of frequency distribution of MLST clonal complexes in *C. jejuni* isolates from Belgian chicken meat preparations and human diarrheal samples

MLST clonal complex	No. of isolates (frequency [%]) from:	
	Chicken meat (n = 76)	Human diarrhea specimens (n = 40)
Not assigned	18 (23.7)	4 (10.0)
CC-21	19 (25.0)	9 (22.5)
CC-42	1 (1.3)	3 (7.5)
CC-45	9 (11.8)	5 (12.5)
CC-48	1 (1.3)	1 (2.5)
CC-52	1 (1.3)	1 (2.5)
CC-206	6 (7.9)	3 (7.5)
CC-257	11 (14.5)	
CC-283		1 (2.5)
CC-353		2 (5.0)
CC-354	4 (5.26)	5 (12.5)
CC-443	3 (3.95)	2 (5.0)
CC-446	1 (1.3)	2 (5.0)
CC-460		1 (2.5)
CC-574	2 (2.63)	
CC-607		1 (2.5)

RESULTS

LOS locus class diversity. The results presented in Table 1 indicate that 87.9% (102/116) of the *C. jejuni* isolates characterized in this study could be assigned to one of the five LOS locus classes (A to E) screened by the class-specific PCR. *C. jejuni* isolates of LOS class A were significantly underrepresented ($P < 0.05$) compared to other classes, while isolates harboring LOS classes C and B represented, together, almost half (48.3% [56/116]) of the total number of isolates screened. LOS classes B and E were significantly ($P < 0.05$) more frequently represented among *C. jejuni* isolates from humans than among those from chicken meat, amounting to 30% (12/40) and 25% (10/40) of the screened enteritis isolates, respectively.

Genotypic concordance between chicken meat and human isolates. MLST identified 34 STs among the 76 *C. jejuni* isolates from chicken meat, with 15.8% (12/76) of the isolates identified as ST-50 (Table 1). Overrepresentation of CC-21 was evident, amounting to one-quarter of all chicken meat isolates (Table 2). *C. jejuni* isolates from human enteritis cases (n = 40) were assigned to 27 STs; 62.9% (17/27) of these STs were singletons, while 4 and 3 isolates were assigned to ST-354 and ST-137, respectively (Table 1). In agreement with the results for chicken meat isolates, CC-21 was slightly more frequently represented than other clonal complexes in *C. jejuni* isolates of human origin (Table 2). *C. jejuni* isolates assigned to CC-45, CC-206, and CC-443 were found comparably frequently in the chicken and human collections (Table 2). However, CC-257 was well represented in *C. jejuni* isolates from chicken meat but was not recorded for isolates of human origin (Table 2).

Eleven STs were featured in both chicken meat and human *C. jejuni* isolates. One of these, ST-3546, is a novel ST, first reported in this Belgian collection; it was isolated from chicken meat in April 2007 and again from a human diarrheal sample in June 2007 (Table 1). Other STs found in both human and chicken samples were ST-354, -19, -443, -572, -42, -48, -50, -53, -122, and -775. Thus, in total, 17 of the 40 (42.5%) *C. jejuni* isolates from human diarrheal samples were found to share STs with isolates from chicken meat preparations.

MLST and PFGE typing versus LOS PCR classes. The results presented in Fig. 1 show a concordance between the *C. jejuni* LOS locus classes assigned by PCR and certain MLST clonal complexes. Of note, a majority (85.7% [24/28]) of *C. jejuni* isolates assigned to CC-21 were found to express LOS locus class C. The correlation between LOS class C and CC-21 was evident in both the human and the chicken collection (Table 1). LOS class B was found in eight different clonal complexes, most frequently in CC-206 (34.6% [9/24]) (Table 1; Fig. 1). In addition, 78.5% (11/14) of *C. jejuni* isolates in CC-45 expressed LOS class E, and 88.9% (8/9) of CC-354 isolates matched PCR assignment to LOS class D (Table 1). The correlations between LOS class D and CC-354 and between LOS class E and CC-45 were evident in both the human and chicken collections.

We were interested in obtaining further insight into the correlation between sequence typing and LOS PCR assignment by using PFGE typing. Figure 2 shows that the correlation between sequence typing results and LOS PCR classifications can be further elaborated by PFGE. For example, and as indicated by MLST (Table 1), *C. jejuni* isolates assigned to LOS class B were relatively diverse; they were grouped by PFGE into two main clusters delineated by a 60% band similarity cutoff (Fig. 2.I). On the other hand, LOS class C isolates were grouped in three very well correlated clusters, among which was a cluster of eight isolates with identical band patterns (Fig. 2.II). These eight isolates were sampled over six different months and originated from four different companies and various slaughter batches (Table 1).

Invasion potential in relation to LOS class, strain source, and genotypic diversity. Fifty-one of the 52 *C. jejuni* isolates tested for invasion potential were able to be internalized into Caco-2 cells; the invasion percentages of these isolates ranged from 0.0002% to 0.26% (Table 3). Thirteen of the 14 *C. jejuni* isolates for which ≥0.1% of the inoculum was internalized expressed sialylated LOS classes (A, B, or C). CC-21, followed by CC-206, was the most frequently represented clonal complex among these 14 isolates (Table 3). There was no significant correlation (P, 0.381 by negative binomial regression anal-

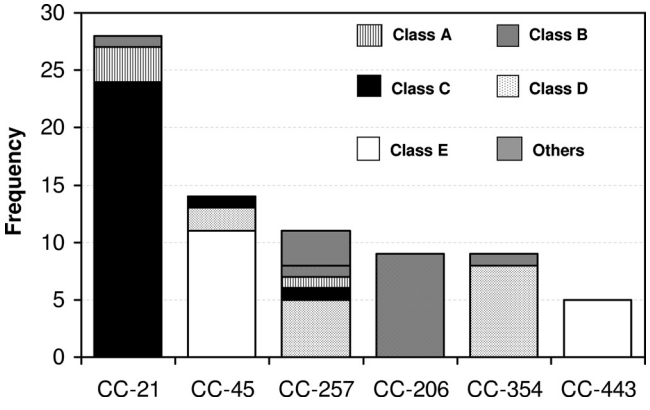


FIG. 1. Frequency distribution of *C. jejuni* LOS classes in correlation with selected MLST clonal complexes found for both chicken meat and human isolates.

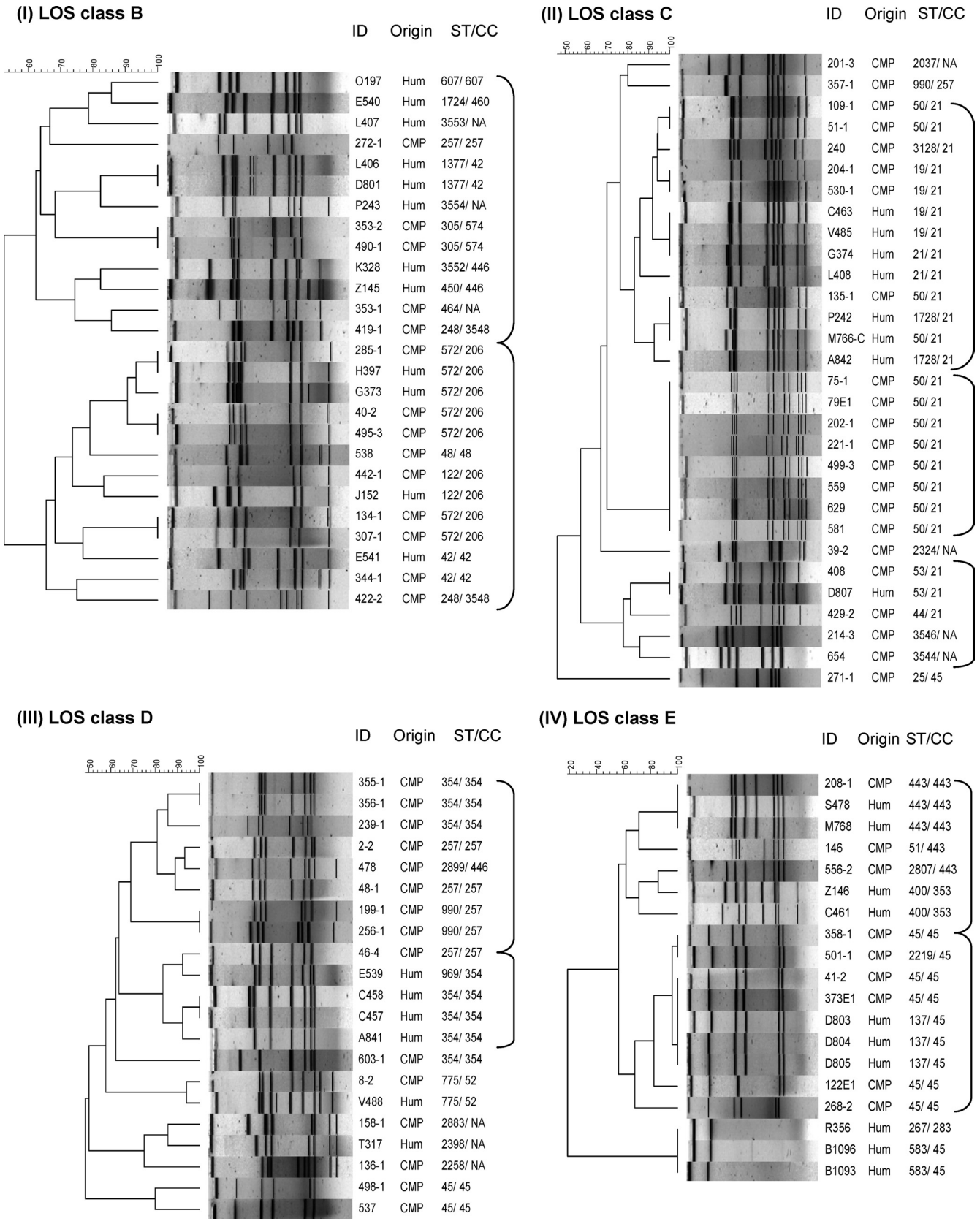


FIG. 2. PFGE dendrogram based on band patterns of SmaI-digested DNA from *C. jejuni* isolates in relation to their LOS assignments. PFGE clusters are marked by curved arcs and presented in correlation with strain identification (ID) numbers, origins (human [Hum] or chicken meat preparation [CMP] samples), and STs and clonal complexes (CC) by MLST.

TABLE 3. Invasion phenotypes of 52 *C. jejuni* strains assayed in Caco-2 cells in relation to their LOS locus classes and MLST genotypes[a]

Isolate no.	Strain	LOS class	Origin	Sequence type	Clonal complex	% of inoculum internalized (avg ± SD)
1	442-1	B	Chicken	ST-122	CC-206	0.2614 ± 0.072
2	344	B	Chicken	ST-42	CC-42	0.2476 ± 0.008
3	353-1	B	Chicken	ST-464	NA[c]	0.2060 ± 0.061
4	H397	B	Human	ST-572	CC-574	0.2031 ± 0.053
5	408	C	Chicken	ST-53	CC-21	0.1811 ± 0.023
6	499-3	C	Chicken	ST-50	CC-21	0.1686 ± 0.034
7	D803	E	Human	ST-137	CC-45	0.1395 ± 0.021
8	G374	C	Human	ST-21	CC-21	0.1293 ± 0.029
9	135-1	C	Chicken	ST-50	CC-21	0.1277 ± 0.014
10	79-E1	C	Chicken	ST-50	CC-21	0.1267 ± 0.022
11	501-3	A	Chicken	ST-883	CC-21	0.1068 ± 0.050
12	490-1	B	Chicken	ST-305	CC-206	0.1051 ± 0.015
13	285-1	B	Chicken	ST-572	CC-206	0.0880 ± 0.025
14	L408	C	Human	ST-21	CC-21	0.0818 ± 0.028
15	187-1	A	Chicken	ST-3550[b]	CC-21	0.0791 ± 0.012
16	C457	D	Human	ST-354	CC-354	0.0790 ± 0.006
17	495-3	B	Chicken	ST-572	CC-206	0.0639 ± 0.009
18	C458	D	Human	ST-354	CC-354	0.0624 ± 0.032
19	J152	B	Human	ST-122	CC-206	0.0544 ± 0.023
20	G373	B	Human	ST-572	NA[c]	0.0539 ± 0.005
21	D807	C	Human	ST-53	CC-21	0.0477 ± 0.003
22	P242	C	Human	ST-1728	CC-21	0.0463 ± 0.018
23	L406	B	Human	ST-1377	CC-42	0.0352 ± 0.002
24	R357	A	Human	ST-262	CC-21	0.0331 ± 0.007
25	R356	E	Human	ST-267	CC-283	0.0306 ± 0.016
26	307-1	B	Chicken	ST-572	CC-206	0.0300 ± 0.012
27	S478	E	Human	ST-443	CC-443	0.0300 ± 0.001
28	501-1	E	Chicken	ST-2219	CC-45	0.0288 ± 0.017
29	358-1	E	Chicken	ST-45	CC-45	0.0258 ± 0.009
30	208	E	Chicken	ST-443	CC-443	0.0224 ± 0.001
31	239-1	D	Chicken	ST-354	CC-354	0.0184 ± 0.002
32	272-2	A	Chicken	ST-257	CC-257	0.0175 ± 0.006
33	2	D	Chicken	ST-257	CC-257	0.0170 ± 0.001
34	530	C	Chicken	ST-19	CC-21	0.0156 ± 0.001
35	V488	D	Human	ST-775	CC-52	0.0143 ± 0.002
36	373	E	Chicken	ST-45	CC-45	0.0124 ± 0.001
37	M768	E	Human	ST-443	CC-443	0.0115 ± 0.002
38	8	D	Chicken	ST-775	CC-52	0.0106 ± 0.001
39	A841	D	Human	ST-354	CC-354	0.0101 ± 0.004
40	46	D	Chicken	ST-257	CC-257	0.0097 ± 0.001
41	157-1	A	Chicken	ST-704	NA	0.0088 ± 0.004
42	E541	B	Human	ST-42	CC-42	0.0065 ± 0.002
43	355-1	D	Chicken	ST-354	CC-354	0.0062 ± 0.004
44	D805	E	Human	ST-137	CC-45	0.0052 ± 0.001
45	603	D	Chicken	ST-354	CC-354	0.0052 ± 0.001
46	C463	C	Human	ST-19	CC-21	0.0051 ± 0.001
47	199	D	Chicken	ST-990	CC-257	0.0030 ± 0.001
48	B1096	E	Human	ST-583	CC-45	0.0024 ± 0.001
49	559-3	C	Chicken	ST-50	CC-21	0.0019 ± 0.001
50	146	E	Chicken	ST-51	CC-443	0.0010 ± 0.002
51	M766C	C	Human	ST-50	CC-21	0.0002 ± 0.002
52	41-2	E	Chicken	ST-45	CC-45	0.0000

[a] Results are given in descending order according to the percentage of the starting viable inoculum internalized into cells after gentamicin resistance assays. The space after isolate 14 marks the cutoff of 0.1% of the inoculum internalized (the standard deviation is considered for isolates 13 and 14).
[b] Novel sequence type.
[c] NA, not assigned to a defined clonal complex (MLST online database last accessed in September 2008).

ysis) between the source of isolates (human or chicken) and the level of invasion of Caco-2 cells (Fig. 3A). The invasion potential of *C. jejuni* isolates of LOS class B was significantly higher than that for other classes (coefficient, 1.66; $P < 0.0001$), followed by *C. jejuni* isolates of LOS class C (coefficient, 1.28; $P < 0.005$) (Fig. 3B). Thus, the invasion potential of *C. jejuni* isolates with sialylated LOS ($n = 29$; classes A, B, and C) was significantly higher ($P = 0.002$) than that of *C. jejuni* isolates with nonsialylated LOS ($n = 23$; classes D and E).

Further characterization of the 52 isolates included in the invasion assay was based on PFGE typing. Figure 4 shows that 50 of these isolates (the other 2 were not restricted by SmaI) can be assigned to three PFGE clusters (P1, P2, and P3). Twenty-two of the 23 *C. jejuni* isolates in PFGE cluster P2 were assigned to the three sialylated LOS classes (Fig. 4). Of note, 10 of the 14 isolates for which ≥0.1% of the inoculum was internalized by Caco-2 cells (Table 3) were in PFGE cluster P2, regardless of their origins.

Figure 5 shows the differences among the invasion potentials of *C. jejuni* isolates assigned to the four main clonal complexes

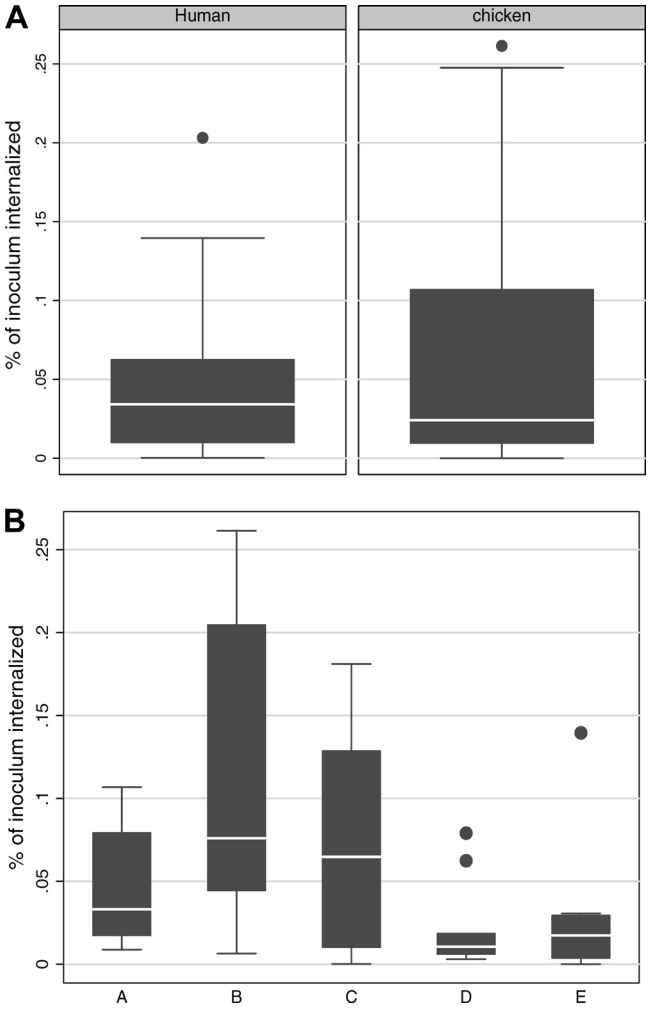
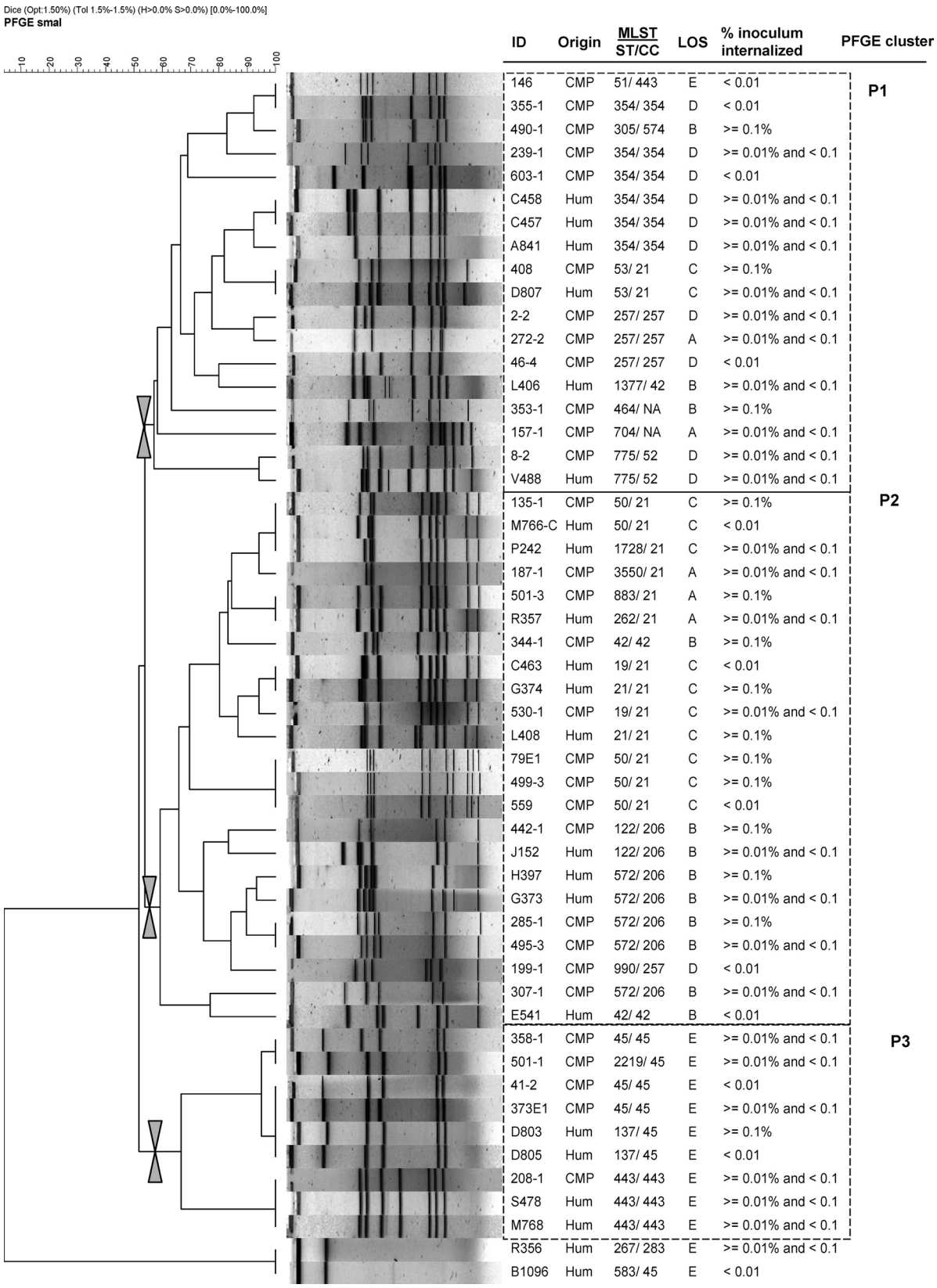


FIG. 3. Differences in the abilities of *C. jejuni* strains ($n = 52$) to invade Caco-2 cells in relation to the sources of the strains (A) and their LOS classes (B). The lines inside the boxes denote the median percentages of internalization of the inocula, and the upper and lower limits of the error bars signify the 75th and 25th percentiles, respectively. The circles above the boxes represent strains with percentages of internalization higher than the 90th percentile.



ID	Origin	MLST ST/CC	LOS	% inoculum internalized	PFGE cluster
146	CMP	51/ 443	E	< 0.01	P1
355-1	CMP	354/ 354	D	< 0.01	
490-1	CMP	305/ 574	B	>= 0.1%	
239-1	CMP	354/ 354	D	>= 0.01% and < 0.1	
603-1	CMP	354/ 354	D	< 0.01	
C458	Hum	354/ 354	D	>= 0.01% and < 0.1	
C457	Hum	354/ 354	D	>= 0.01% and < 0.1	
A841	Hum	354/ 354	D	>= 0.01% and < 0.1	
408	CMP	53/ 21	C	>= 0.1%	
D807	Hum	53/ 21	C	>= 0.01% and < 0.1	
2-2	CMP	257/ 257	D	>= 0.01% and < 0.1	
272-2	CMP	257/ 257	A	>= 0.01% and < 0.1	
46-4	CMP	257/ 257	D	< 0.01	
L406	Hum	1377/ 42	B	>= 0.01% and < 0.1	
353-1	CMP	464/ NA	B	>= 0.1%	
157-1	CMP	704/ NA	A	>= 0.01% and < 0.1	
8-2	CMP	775/ 52	D	>= 0.01% and < 0.1	
V488	Hum	775/ 52	D	>= 0.01% and < 0.1	
135-1	CMP	50/ 21	C	>= 0.1%	P2
M766-C	Hum	50/ 21	C	< 0.01	
P242	Hum	1728/ 21	C	>= 0.01% and < 0.1	
187-1	CMP	3550/ 21	A	>= 0.01% and < 0.1	
501-3	CMP	883/ 21	A	>= 0.1%	
R357	Hum	262/ 21	A	>= 0.01% and < 0.1	
344-1	CMP	42/ 42	B	>= 0.1%	
C463	Hum	19/ 21	C	< 0.01	
G374	Hum	21/ 21	C	>= 0.1%	
530-1	CMP	19/ 21	C	>= 0.01% and < 0.1	
L408	Hum	21/ 21	C	>= 0.1%	
79E1	CMP	50/ 21	C	>= 0.1%	
499-3	CMP	50/ 21	C	>= 0.1%	
559	CMP	50/ 21	C	< 0.01	
442-1	CMP	122/ 206	B	>= 0.1%	
J152	Hum	122/ 206	B	>= 0.01% and < 0.1	
H397	Hum	572/ 206	B	>= 0.1%	
G373	Hum	572/ 206	B	>= 0.01% and < 0.1	
285-1	CMP	572/ 206	B	>= 0.1%	
495-3	CMP	572/ 206	B	>= 0.01% and < 0.1	
199-1	CMP	990/ 257	D	< 0.01	
307-1	CMP	572/ 206	B	>= 0.01% and < 0.1	
E541	Hum	42/ 42	B	< 0.01	
358-1	CMP	45/ 45	E	>= 0.01% and < 0.1	P3
501-1	CMP	2219/ 45	E	>= 0.01% and < 0.1	
41-2	CMP	45/ 45	E	< 0.01	
373E1	CMP	45/ 45	E	>= 0.01% and < 0.1	
D803	Hum	137/ 45	E	>= 0.1%	
D805	Hum	137/ 45	E	< 0.01	
208-1	CMP	443/ 443	E	>= 0.01% and < 0.1	
S478	Hum	443/ 443	E	>= 0.01% and < 0.1	
M768	Hum	443/ 443	E	>= 0.01% and < 0.1	
R356	Hum	267/ 283	E	>= 0.01% and < 0.1	
B1096	Hum	583/ 45	E	< 0.01	

FIG. 4. PFGE dendrogram based on SmaI-digested DNA from 52 *C. jejuni* isolates characterized for their potential to invade Caco-2 cells. PFGE clusters (P1, P2, and P3) are grouped in boxes with dashed outlines and are presented in correlation with strain identification (ID) numbers, origins (human [Hum] or chicken meat preparation [CMP] samples), STs and clonal complexes (CC) by MLST, LOS classes, and percentages of inocula internalized in Caco-2 cells.

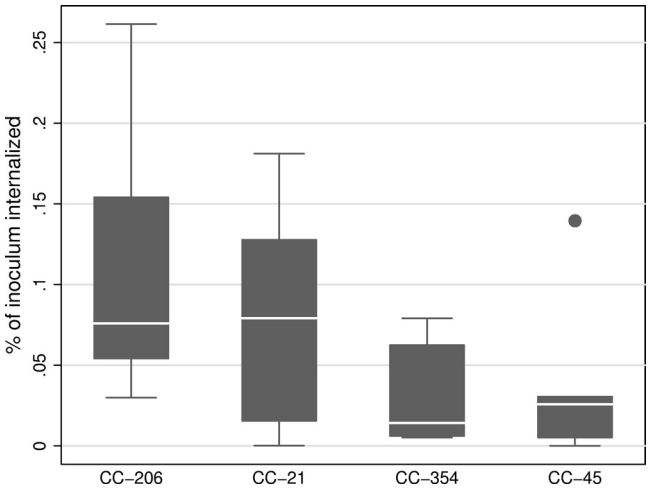


FIG. 5. Differences in invasion potential among *C. jejuni* isolates from the four main clonal complexes found in both chicken and human isolates. The lines inside the boxes denote the median percentages of internalization of the inocula, and the upper and lower limits of the error bars signify the 75th and 25th percentiles, respectively. The circle above the CC-45 box represents a strain with an internalization level higher than the 90th percentile.

found in the chicken meat and human collections: CC-21, CC-45, CC-206, and CC-354 (Table 2). The invasion potential of *C. jejuni* isolates assigned to CC-45 was significantly lower ($P < 0.05$) than those for other clonal complexes. There was no significant difference between CC-206 and CC-21 isolates ($P > 0.05$), but the invasion level of *C. jejuni* isolates in each of CC-206 and CC-21 was significantly higher ($P < 0.05$) than those of isolates in both CC-354 and CC-45. In the comparison between clonal complexes, the number of isolates per clonal complex was selected in proportion to its frequency (Table 2) and in view of LOS PCR results (Table 1). For example, 15 of the 28 *C. jejuni* isolates in CC-21 were assayed for invasion and distributed as 12 of the 24 isolates identified with LOS class C plus the 3 isolates identified with LOS class A (Table 3).

DISCUSSION

***C. jejuni* LOS class diversity.** The present study adds to the limited knowledge about LOS class diversity in *C. jejuni* isolates from chicken meat and about the invasion potential of such isolates compared to those from human diarrheal samples. In the present study, PCR screening targeted only five specific classes, despite recent increases in the number of LOS locus classes (43). Nevertheless, a majority (87.1% [101/116]) of the isolates screened were members of these five classes, and 52.6% of *C. jejuni* isolates from Belgian chicken meat and human diarrheal samples possessed sialylated LOS (class A, B, or C).

It is estimated that 0.3 in 1,000 to 1 in 1,000 *Campylobacter* infections leads to GBS. In addition, epidemiological studies have indicated that a *C. jejuni* infection precedes GBS in 20 to 50% of cases in Europe, North and South America, Japan, and Australia (27, 46). *C. jejuni* strains expressing class A and B LOS loci have been associated with postinfection neuropathy (18). In the present study, these two classes together were found in almost one-quarter of all chicken meat isolates (Table 1): 5.3% (4/76) and 18.4% (14/76) of *C. jejuni* isolates from the chicken meat collection were found to express LOS classes A and B, respectively. In a previous study, we showed that the prevalence of *Campylobacter* spp. in Belgian chicken meat preparations, including isolates used in the present study, is around 60% (22). The high prevalence of *Campylobacter* spp. in chicken meat, combined with the fact that a substantial subset of the *C. jejuni* isolates characterized in this study possess neuropathy-associated LOS, can be regarded as a worrying signal. Therefore, strategies to control *Campylobacter* contamination of chicken meat might reduce the morbidity due to GBS, in addition to reducing the level of *Campylobacter*-related human enteric illnesses.

Genotyping and LOS class assignment. Human isolates included in the present study were isolated between May and September 2007. Epidemiological evidence from many countries (38, 40) suggests that human campylobacteriosis tends to increase during this period of the year. Using MLST, we showed that *C. jejuni* strains isolated from human diarrheal samples during this period exhibit considerable genetic overlap (42.5% [17/40] of human isolates) with isolates from the chicken meat population. In addition, the distribution of MLST clonal complexes showed good concordance between chicken and human isolates (Table 2); however, CC-21 was more frequently represented than other clonal complexes in both. CC-21 is the largest complex in the general population structure of *C. jejuni* (11); it is widespread in multiple hosts and has previously been reported to be associated with infections of humans and with livestock and environmental sources, such as chicken, cattle, contaminated milk, and water (11, 47). Molecular epidemiological evidence suggests that this clonal complex is associated with environmental and food-borne transmission (8, 47). Considering the possible epidemiological significance of CC-21, Best et al. (4) described a single-nucleotide polymorphism analysis assay enabling rapid strain profiling for CC-21. In the present study, we showed that PCR screening of *C. jejuni* LOS class C could correctly predict CC-21 for 86% of isolates screened (Table 1). Additionally, comparable correlations were evident in other LOS class–MLST clonal complex combinations (Fig. 1). Thus, PCR screening for *C. jejuni* LOS classes could be of value in population structure studies, especially for elaborating the clonal relationships between *C. jejuni* isolates.

The *C. jejuni* isolates included in this study were selected in such a way as to generate an epidemiologically diverse collection, by including isolates of human and chicken origins, cultured over a period of 10 months. Moreover, the chicken meat collection contained isolates from five different producers and from a variety of processing batches (Table 1). Thus, for example, the correlation between the dominant clonal complex CC-21 and LOS locus class C (Fig. 1) is unlikely to be due to bias or chance. Parker et al. (42) indicated that LOS class C was detected in *C. jejuni* isolates from all sources, based on PCR screening of a collection of 123 clinical and environmental strains. In addition, Müller et al. (36) found that most *C. jejuni* isolates from human and turkey sources express primarily LOS class C. These studies, in addition to our PCR screening and MLST data, suggest a possible role of LOS class C in the evolution of the widely spread clonal complex CC-21 that

might be of particular importance in the poultry meat-related transmission of *C. jejuni* to humans. The correlation between *C. jejuni* with sialylated LOS class C and the ecologically diverse CC-21 could be an example of an adaptive strategy used by *C. jejuni* to modulate cell surface carbohydrate structures in order to better survive in a given host species. However, further screening studies are needed to confirm our hypothesis regarding such presumed correlations.

The correlation between certain MLST clonal complexes and LOS PCR assignment was further elaborated using PFGE. *C. jejuni* isolates of LOS classes A, B, and C were grouped into one PFGE cluster (P2) (Fig. 4), indicating a phylogenetic correlation between isolates harboring these sialylated classes. In fact, our results (Fig. 4) show that *C. jejuni* LOS classes A and C were actually sharing the same MLST clonal complex (CC-21). Recombination between locus class C and class A can occur between regions of homologues that flank these LOS biosynthesis loci and has been reported previously for *C. jejuni* strain GB11 (15). Moreover, it is believed that LOS locus class B could be an evolutionary intermediate between classes A and C (15), which could explain our finding of close phylogenetic correlation between *C. jejuni* isolates with LOS class B and those with classes A and C. Thus, the phylogenetic correlation between LOS classes A, B, and C can be attributed to the nature of the LOS loci of *C. jejuni* as hot spots for genetic exchange.

Invasion potentials and LOS locus class variations. The results from our invasion assays support the growing hypothesis that the enhanced invasiveness of *C. jejuni* strains with sialylated LOS could contribute to postinfectious complications. Perera et al. (44) previously showed that the presence of intact LOS is vital for *C. jejuni* adherence to and invasion of INT-407 cells. In addition, our results confirm the recent finding by Louwen et al. (34) that *C. jejuni* isolates with sialylated LOS exhibit a higher invasion potential than *C. jejuni* isolates with nonsialylated LOS (classes D and E). Their conclusion was based on *C. jejuni* strains isolated only from human patients with enteritis and GBS, whereas in the present study we extend the same conclusion to *C. jejuni* isolates from ready-to-cook chicken meat. Of note, we used the same invasion assay protocol as that used by Louwen et al. (34), in order to ensure a valid comparison of their and our results.

In the present study, no significant differences in the invasion phenotype were found between *C. jejuni* isolates from patients with diarrhea and *C. jejuni* isolates from chicken meat meant for human consumption. Previous studies correlating invasion phenotypes with the sources of isolates provided contradictory findings; indeed, many of these studies concluded that the invasiveness of clinical strains is higher than that of strains isolated from poultry (12, 13, 37, 39, 51). However, some studies indicate no difference in invasion, or in adhesion, between *C. jejuni* isolates from human and poultry sources (14, 33). Nevertheless, the ability of *C. jejuni* to invade epithelial cells in vitro is recognized as being strain dependent (5, 25, 31). Our results support such a concept to a certain extent; for example, the average invasion potential of *C. jejuni* isolates with sialylated LOS of class B was significantly higher than that for other classes (Fig. 3B). However, some of these isolates still exhibit variant invasion phenotypes (Table 3). On the other hand, the majority of *C. jejuni* isolates with nonsialylated LOS (classes D and E) were associated with a relatively lower invasion potential than strains expressing sialylated LOS classes (Fig. 3B). Among *C. jejuni* isolates expressing LOS class D, human isolates showed higher invasion levels (Table 3) than chicken meat isolates, despite sharing the same ST. Presumably a host adaptation effect was behind this finding.

Invasiveness results from the interplay of numerous bacterial and host factors. PCR screening of seven virulence-associated genes indicated the presence of *ceuE*, *cadF*, *ciaB*, *pldA*, *cdtA*, *cdtB*, and *cdtC* in all strains (data not shown). In addition, we showed that isolates with a common genotypic profile, as identified by MLST clonal complexes and PFGE similarity clusters, might have common in vitro virulence characteristics as well (Fig. 4); *C. jejuni* isolates of CC-21 and CC-206 were associated with a high invasion potential, while isolates of CC-45 were less invasive (Fig. 4). Clearly there are considerable discrepancies between studies attempting to correlate invasiveness, or other virulence attributes, with certain genotypes of *C. jejuni*. The lack of a common nomenclature for genotype assignment makes it difficult to use these studies to establish a correlation between typing data for *C. jejuni* and a selected virulence trait. Studies using MLST could be of value in solving such dilemma, since the technique uses a robust standard numerical assignment of STs and clonal complexes. Recently, Fearnley and colleagues (12) studied the invasion of INT-407 cells by 113 *C. jejuni* strains. They found that four of five "hyperinvasive" *C. jejuni* strains were associated with CC-21, but their study did not identify a correlation between MLST clonal complexes and a specific pattern of invasion. Alternatively, Hänel et al. (23) identified an association between the PFGE genotypes of 17 *Campylobacter* isolates from turkeys and their invasion phenotypes in Caco-2 cells. It should be noted that in our study, the strains selected for the study of invasiveness were found to be inherently associated with certain LOS classes (Fig. 2). However, this association seems to be a function of a biological correlation between *C. jejuni* with sialylated/nonsialylated LOS classes and certain genotypes. To the best of our knowledge, the present study is the first to highlight such a possible biological correlation, and we show an evident impact of such a correlation on the invasion potential of *C. jejuni* strains.

In conclusion, our results support the growing scientific evidence that sialylation of LOS could be advantageous for the fitness and infectivity potential of *C. jejuni* in different reservoirs and hosts. The present study revealed a correlation between MLST clonal complexes and certain LOS locus classes. This correlation needs to be investigated further, possibly to determine if it underlies a biological advantage for *C. jejuni* in colonizing birds and surviving in the environment.

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